IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE US PATENT APPLICATION OF
YIQING ZOU ET AL.
SERIAL NO. 08/216,440
FILED: March23, 1994
FOR: ANTIMALARIAL COMPOSITIONS

Commissioner of Patents and Trademarks
Washington D.C. 20231 USA

DECLARATION OF WALTHER H. WERNSDORFER UNDER RULE 132

I, Walther H. Wernsdorfer, citizen of the Federal Republic of Germany and resident of Vienna, Austria, do hereby declare and say as follows:

That I am a Graduate of The Friedrich Alexander University of Erlangen, Federal Republic of Germany, where I graduated in 1952 and obtained the approbation in medicine (M.B.B.S);

That I am a Graduate of The Ludwig Maximilian University of Munich, Federal Republic of Germany, where I graduated in 1953 and obtained the Degree of a Doctor of Medicine (M.D.);

That I have undergone postgraduate training in tropical medicine at the Swiss Tropical Institute in Basel, Switzerland, and obtained in 1952 the Diploma of Tropical Medicine (D.T.M.);

That I have undergone postgraduate training in public health at the University of Bristol, U.K., and obtained in 1967 the Diploma of Public Health (D.P.H.);

That, as from 1958 until 1988, I have served the World Health Organization as a staff member in the fields of tropical medicine and malaria; between 1978 and 1988 as Chief Medical Officer in charge of global malaria research and *ex officio* Secretary of the Scientific Working Groups on the Chemotherapy and Immunology of Malaria, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases;

That, as from 1960, I held academic teaching assignments in addition to my WHO assignments, with the Faculty of Medicine, University of Khartoum, Sudan, the University of Tunisia, and the Université Claude Bernard, Lyon, France;

That, in 1988, I have been appointed visiting professor at the University of Vienna, Austria, and the Universiti Sains Malaysia, Penang, and in 1993 at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand;

That I am the principal author or coauthor of approximately 100 publications, mainly in the field of malaria and malaria chemotherapy;

That I am a registered member of the medical profession (Medical Board of Central Franconia, Federal Republic of Germany);

That I am a member of the following professional bodies/organizations:

World Health Organization (WHO) Expert Panel on Malaria
German Society of Tropical Medicine (Honorary Member)
Swiss Society of Tropical Medicine and Parasitology (Honorary Member)
Austrian Society of Tropical Medicine and Parasitology (Council Member)
Royal Society of Tropical Medicine and Hygiene (U.K.)
British Society of Public Health
British Society of Parasitology;

That I am presently working as Visiting Professor (Tropical Medicine) at the Institute for Specific Prophylaxis and Tropical Medicine, Faculty of Medicine, University of Vienna, Austria, and Visiting Professor at the National Centre for Drug Research, Universiti Sains Malaysia, Penang, Malaysia (Tropical Clinical Pharmacology), and as Visiting Professor at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (Tropical Clinical Pharmacology);

That I have reviewed the Chemical Abstracts Reference 103:134524 which was cited by the U.S. Patent Office;

That I have reviewed the English translation of the reference Yaoxue Xuebao (1985), 20 (3), 211 - 213 ("The Reference") to which the Chemical Abstracts Reference 103:134524 refers;

That page 1, paragraph 1, line 3 of The Reference incorrectly refers to oral administration of artemether;

That the performed mode of administering to mice the therapeutic agents artemether and chloroquine is in reality intragastric gavage;

That the in-vivo experiments mentioned in The Reference and the conclusions drawn therefrom are as follows:

That the in-vivo experimentation does not correspond to any established pharmacological model in malariology;

That no base levels of IgG have been mentioned in Sections 1-3 and no base values of spleen weight have been mentioned in Section 4;

That the observation of a response related to serum IgG or change of spleen weight would only be conclusive if base values at the beginning of experimentation were given;

That the observation of immunological phenomena such as the formation or reduction of serum IgG would only be conclusive if therapeutic doses were administered;

That the dose of 200 mg/kg twice per day and 100 mg/kg twice per day administered exceeds the therapeutic doses of artemether by a factor of about 50 to 100 (Exhibit 1): ED_{90}: 5.3 mg/kg (p.o.)

That the administration of a high amount of artemether results in cytotoxic effects by destruction of erythrocytes. This renders the results inconclusive; it is not clear whether any observed spleen enlargement is caused by the active agent administered or by the enhanced removal of erythrocytes damaged by the excessive concentrations of the drug;

That the dose of 200 mg/kg twice daily and 100 mg/kg twice daily administered to mice is in the toxic range;

That the toxic dose (LD_{50}) of artemether, which defines the dose causing 50 % lethality, is 263 mg/kg (Exhibit 2);

That the high amounts administered must have resulted in the death of at least some animals which has not been reported in The Reference;

That the high amounts administered reflect the assumption of poor gastrointestinal absorption; poor gastrointestinal absorption of an active agent or active agent composition is not indicative of any suitability for an oral dosage form;

That the poor gastrointestinal absorption of artemether has been assumed in view of the established poor gastrointestinal absorption of artemisinin [= Qinghaosu (Exhibit 3)];

That the i.g. administration of artemether with the suspending agent tragacanth is indicative of the unsuitability of artemether for oral administration by conventional dosage forms such as tablets;

That the choice of tragacanth as a suspending agent is indicative of the low water solubility of the active agent artemether (Exhibit 4);

That the immune suppression as shown by the reduction of serum IgG-levels in Sections 1 and 2 or the absence of an immune response according to Section 3 are explicable by specific cytotoxic effects of artemether administered in high amounts; resulting in a reduction of IgG levels in the absence of new formation as the productive cells are damaged;

That the increase of spleen weight in malaria-free normal mice (Section 1) and SRBC immunized mice (Section 2) is explicable by the hypertrophy of the spleen caused by erythrotoxic effects of the high amounts of artemether administered;

That the experimentation is defective as to the different time periods for observation, i.e. seven days in Sections 1 and 2 and four days in Section 3, which allows no direct comparison of the results obtained;

That the different time periods for observation result from a clear inconsistency in experimentation; the active agent artemether has been administered to malaria-free normal mice (Section 1) and SRBC immunized mice (Section 2) twice daily for seven days, whereas according to Section 3 (in Plasmodium berghei infected mice) the active agent artemether has been administered twice daily only for four days; no reasonable explanation has been given for this inconsistency;

That this inconsistency allows no direct comparison of the effect of artemether on serum IgG in malaria-free normal mice (Section 1) and SRBC immunized mice (Section 2) as compared to the effect of artemether on serum IgG in Plasmodium berghei infected mice in Section 3;

That the shorter time period for administering the active agent artemether to Plasmodium berghei infected mice (Section 3) explains the fact that no difference in serum IgG level of treated infected animals has been observed as compared to the untreated infected control group;

That a lowering of the IgG level would have been observed in the event that the active agent artemether had also been administered to Plasmodium berghei infected mice for seven days;

That the same inconsistency in experimentation mentioned above also renders inconclusive the results according to Section 4 (effect of artemether on spleen weight): the increase of the spleen weight in malaria-free normal mice (Section 1) and SRBC immunized mice (Section 2) is explicable by the erythrotoxic effects of the high dose amounts of artemether administered and the consequent response by enlargement of the spleen as the organ where the removal of damaged erythrocytes takes place; a similar increase of the spleen weight due to cytotoxic effects of the high amounts administered would have also been observed in infected mice if the observation period had correctly been extended to seven days in agreement with the experimentation according to Sections 1 and 2;

That the lower spleen weight observed in Plasmodium berghei infected mice is explicable by the action of artemether on parasite infection which is faster than the erythrocyte destruction in non-infected animals;

That in view of the excessive and toxic dose regimens used, the experimentation does not at all reflect any immunological changes which would occur with therapeutic doses of artemether;

Conclusion

The interpretation of the drug effects on IgG levels and spleen weight in The Reference is not tenable in the absence of appropriate haematological and cytological investigations, including the histological examination of the spleen;

The immune suppression as substantiated by the reduction of serum IgG-levels in Sections 1 and 2 and the absence of an immune response according to Section 3 reflect a general undesirability of administering artemether via the gastrointestinal tract. When immunological effects are considered, only immune stimulation may be desirable when administering antimalarial agents.
The in-vivo experiments carried out according to The Reference do not relate to the therapeutic use of artemether.

The experiments do not suggest an oral dosage form wherein the active agent artemether has been formulated.

The Undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issueing thereon.

The following exhibits are part of the Declaration:

Exhibit 1: Journal of Traditional Chinese Medicine 2(1): 17-24, 1982
Exhibit 2: Journal of Traditional Chinese Medicine 2(1): 31-38, 1982
Exhibit 3: W. H. Wernsdorfer, P.I . Trigg: Malaria Principles and Practice of Malariology, Chapter51: Recent progress of malaria research: chemotherapy, pages 1618 - 1619
Exhibit 4: Martindale, The Extra Pharmacopoeia, 31st Ed. 1996, pg.1541

Signed at Vienna this 16TH day of DECEMBER 1996

Walther H. Wernsdorfer

$\log 1/C = 1.202 - 0.1983(\log P)^2 + 1.549 \log P - 0.6125\ I_{a,\beta}$
$n=49;\ r=0.9111;\ s=0.153;\ \log P_0 = 2.91$
$t_1=6.083;\ t_2=12.782$

Regression of the data of 14 ethereal compounds gave the following equation:

$\log 1/C = 0.9575 - 0.2231(\log P)^2 + 1.1620 \log P - 0.637\sigma^* - 0.1451 \cdot I_{a,\beta}$
$n=14;\ r=0.906;\ s=0.168;\ \log P_0 = 2.60$
$t_1=2.549;\ t_2=2.118;\ t_3=5.272;\ t_4=1.886$

Correlation coefficient r and standard deviation s values point to parabolic correlation between the lipophilicity and the biological activity. The ideal lipophilic character is ealculated to be:

$\log P_0 = 2.6 - 2.9$

However, in the series of ethereal compounds, the electronic parameters of various substituents also significantly affect the antimalarial activity of the compounds. Such results could prove useful for designing new compounds in future.

A few compounds prepared in the present study were chosen for extensive pharmacological and toxicological examinations, and subsequently sent for clinical evaluation. Preliminary clinical trials gave promising results.

ACKNOWLEDGMENTS

Part of this investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1. Qinghaosu Antimalaria Coordinating Research Group: Antimalaria Studies on Qinghaosu. Chinese Medical Journal, 92:861, 1979.

2. Hofle, G. et al: 4-Dialkylaminopyridines as highly active acylation catalysts, Angewandte Chemie, International Edition, 17:569, 1978.

Abstracts

EXPLORATION OF THE NATURE AND CLINICAL SIGNIFICANCE OF THE TCM THEORY THAT THE LUNG IS EXTERIO-INTERIORLY RELATED TO THE LARGE INTESTINE

(Presented at the First Symposiom of Chinese Association for the Integration of Traditional and Western Medicine)

Wang Jinda 王今达 et al.
First Central Hospital, Tianjin

TCM holds that the lung is closely connected with the large intestine. This paper aims to explore the intrinsic nature of the theory by clinical analysis and experimental investigation.

Analysis of 48 cases of ARDS (acute respiratory dyspnea syndrome) admitted Jan-July 1978 and caused by various pathogenic factors showed 25(52.1%) manifesting serious disturbances of intestinal function. This suggested that the etiology of ARDS is closely related to such severe intestinal functional disorders as perforation and intestinal necrosis following volvulus, conditions which as a rule precede the occurrence of lung lesions. The intrinsic correlation might be intestinal toxins derived from the intestine and this, in turn, might be the nature of the above TCM theory. Meanwhile, improvement of intestinal function might lead to the relief of lung injury, in which case TCM theory reflects objective findings.

On the basis of the above analysis, animal ARDS models are performed by ligating the superior mesenteric arteries in 2 groups of normal rabbits: one of 20 animals with arteries ligated, the other of 7 animals with the arteries ligated +estimation of endotoxin in the blood. A third, control group included 10 rabbits. The results were that the first group all showed lung injury with endotoxemia developing 90 minutes after ligation of the vessels, while the control group showed no lung lesions at all, illustrating that ischemic intestinal lesion is intrinsically and definitely related to lung lesions. The conclusion is logical that endotoxni is one of the causes of lung lesion, termed "feverish toxicity" in TCM. Another possible cause of lung injury is stagnation of extravasated blood in the intestinal tract as a result of interruption of blood circulation. The conclusion may therefore be reached that the theory that the lung is exterio-interiorly related to the large intestine corelates somewhat to insuffcient blood circulation of intestinal tract and endotoxin of the intestine (stagnant blood in the intestine and reverish toxicity in TCM) in Western medicine.

Journal of Traditional Chinese Medicine 2(1):17-24, 1982.

(7)

ANTIMALARIAL EFFICACY AND MODE OF ACTION OF QINGHAOSU AND ITS DERIVATIVES IN EXPERIMENTAL MODELS

*China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials**

Qinghaosu (QHS) is a pure compound isolated from the Chinese herb Qinghao (Artemisia annua L.). The antimalarial action of this compound and its derivatives artemether and sodium artesunate (804-Na) were evaluated in vivo; also, the efficacy of suppressing the erythrocytic stage of chloroquine (CQ)-sensitive and CQ-resistant strains of P. berghei, the rate of schizonticidal action on the CQ-sensitive strain and effects on the exoerythrocytic stages of P. cynomolgi and other plasmodia. In addition, pharmacological tests were performed in parasitized RBC cultures. Results showed that QHS and its derivatives do affect the erythrocytic stages of plasmodia and CQ-resistant parasites have low cross resistance to them. Their parasiticidal action is faster than that of CQ, but QHS does not affect the exoerythrocytic stages of several plasmodia. The results also showed that structure modification of QHS would be useful in improving the therapeutic effect of QHS. The mode of action of QHS is quite different from that of other types of antimalarial drugs now available, as indicated by preliminary studies on P. knowlesi or P. berghei in vivo and P. falciparum in vitro.

Preliminary screening showed QHS to have good antimalarial action against the trophozoites in the erythrocytic stage of *P. berghei*. Later, a series of QHS derivatives with antimalarial property were synthesized, in which artemether was characterized by high solubility in oil while sodium artesunate was highly soluble in water. In order to evaluate the antimalarial action of these, experimental studies were undertaken in vitro and in vivo in animals infected with avian, rodent and primate malaria parasites. The mode of antimalarial action of the derivatives was explored and preliminary results were obtained.

ANTIMALARIAL ACTION OF QHS AND ITS DERIVATIVES

Effect on Erythrocytic Stage of P. berghei

1. Determination of SD_{50} and SD_{90} against CQ-sensitive strain.

Hybrid Shanghai mice, 18-22 g, were inoculated with 5×10^6 infected RBC intraperitoneally on D_0. Drugs were given once a day from D_1 to D_3. Blood smears were made on D_4, stained and examined under microscope. SD_{50} and SD_{90} were calculated by the simpli-

* Main research units:

1. Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine
2. Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
3. Shanghai Institute of Materia Medica, Chinese Academy of Sciences
4. Guangxi Medical College
5. Guangxi Traditional Chinese Medical College
6. Guangzhou College of Traditional Chinese Medicine
7. Second Military Medical College, Shanghai
8. Institute of Parasitic Disease, Chinese Academy of Medical Sciences

*Table 1. Therapeutic effect of QHS, artemether, sodium artesunate and CQ on CQ-sensitive strain of P. berghei**

Drug	Preparation	Route of admin-istration	SD_{50} (MKD)	SD_{90} (MKD)
QHS	Water suspension	ig	10.8	28.3
	Water suspension	im	4.90	8.01
	Oil suspension	im	0.77	2.15
Artemether	Oil solution	im	0.37	0.53
Sodium artesunate	Water solution	im	0.54	1.77
	Water solution	iv	0.94	3.10
CQ†	Water solution	ig	1.85	2.60
	Water solution	im	0.60	1.12
	Water solution	iv	0.67	1.25

* Inoculation on D_0, drug given once daily on D_1-D_3, blood smear made on D_4.

† Chloroquine phosphate was used in the experiments summarized in this review and dosage was calculated as base of CQ.

fied probit analysis. The forms of drug preparations and routes of administration are given in Table 1. CQ was taken as standard drug for comparison. The results showed that the antimalarial action of QHS was greater when given intramuscularly than per os and intramuscular injection of oil preparation gave better results than that of water suspension. Also by intramuscular injection, a smaller dose of the SD_{50} of artemether than of CQ, was given, while those of QHS and sodium artesunate were similar to the amount of CQ (Table 1).

2. Determination of CD_{50} against CQ-resistant strain.

Hybrid Shanghai mice, 18-22 g, were inoculated with 1.5×10^6 infected RBC intraperitoneally on D_0. The resistance index of the CQ-resistant strain of *P. berghei* was over 52. Drugs were given once a day from D_2 to D_4. Blood smears and microscopic examinations were performed on D_{32}. Calculated by Finney's method, the CD_{50} of oil suspension of QHS was 74 MKD (mg/kg/day) and that of artemether was 4.2 MKD. The cure rate of artemether against *P. berghei* was apparently higher than that of QHS.

*Table 2. The parasiticidal rate of QHS, artemether, sodium artesunate and CQ in treating CQ-sensitive strain of P. berghei at equi-effective dosage**

Drug	Preparation	Route of administration	Dosage	No. of mice	b value†	bA/bCQ††	P
QHS	Water suspension	ig	283	10	-0.1353	3.04	$P < 0.001$
	Water suspension	im	26	5	-0.0711	1.60	$P < 0.05$
	Oil suspension	im	5	5	-0.1169	2.63	$P < 0.001$
Artemether	Oil solution	im	5.3	10	-0.0968	2.17	$P < 0.01$
Sodium artesunate	Water solution	im	35.4	10	-0.1930	3.13	$P < 0.001$
	Water solution	iv	62.0	10	-0.1326	2.98	$P < 0.001$
CQ	Water solution	ig	14.5	5	-0.0445	1.00	
	Water solution	im	6.0	5	-0.0458	1.02	$P > 0.05$
	Water solution	iv	6.24	10	-0.1086	2.44	$P < 0.001$

* Equi-effective dosage, i.e. the minimum clearance dosage in treating the rodent malaria

† Slope of the regression line of parasiticidal rate

†† The ratio of b values of each drug(bA) to that of oral CQ (bCQ)

Table 3. Comparison of the effect of QHS, artemether and sodium artesunate normal strain and highly CQ-resistant strain of P. berghei

Drug	Route of administration	Normal strain ED_{50}*	Normal strain SD_{50}*	Resistant strain ED_{50}*	Resistant strain SD_{50}*	Resistance index
QHS†	ig	46.3		176.6		3.8
Artemether††	im	0.72		1.2		1.7
Sodium artesunate†	iv		0.3		0.5	1.7

* Daily dose in mg/kg

† Inoculation on D_0, drug given once daily on D_1-D_3, blood smear made on D_4

†† Inoculation on D_0, drug given once daily on D_7-D_{11}, blood smear made on D_{14}

3. Comparison of the parasiticidal rate of various preparations at equi-effective dosage against CQ-sensitive strain.

Drugs were given when parasitemia reached $5 \pm 2\%$. The minimum clearance dose of each drug, as determined by preliminary trials, was administered once a day for 3 successive days. Blood smears were made every 8 hours after the first dose in order to calculate the percentage of residual parasitemia in each group. It was found that the parasiticidal rates of QHS (water suspension ig or oil suspension im), artemether and sodium artesunate were nearly the same, all being faster than for CQ (Table 2). Eight hours after the first dose of QHS (oil suspension or water suspension) or artemether, the percentage of parasitemia continued to rise to a certain extent while the percentage of parasitized RBC decrease instantly using sodium artesunate. Sodium artesunate was less effective in the late stage, and the clearance of parasitemia was relatively slow and recrudescence occurred sooner.

4. Therapeutic effect against CQ-resistant strain.

ED_{50} or SD_{50} of QHS, artemether and sodium artesunate (effectiveness in a 3-day regime against CQ-resistant strain and CQ-sensitive strain of *P. berghei*) was ascertained and resistance index was calculated. Table 3 shows that highly CQ-resistant strain of *P. berghei* had low cross resistance to these 3 drugs.

Table 4. Therapeutic effect of im injection of QHS and artemether on the erythrocytic stage of P. cynomolgi

Drug	Preparation	Dosage* (MKD)	Time of clearance (day)	Recrudescence
QHS	Oil suspension	20	2	No
		20	2	No
		10	2	On 20th day
		10	2	On 23th day
		4	4	On 8th day
		4	2	On 24th day
		1	3	On 9th day
Artemether	Oil solution	8	2	No
		8	2	No
		4	2	No
		4	1	No
		4	2	On 20th day
		2	1	No
		2	2	No
		1	2	On 16th day

* once daily for 3 days

*Table 5. Effect of ig QHS on exoerythrocytic stage of P. gallinaceum**

Drug	Dosage MKD × days	No. of chickens	Parasitemia		Time of death (day)†
			No. of animal	Day of appearance	
QHS**	200 × 6	5	5	8	10-14
	400 × 6	5	...	...	< 7
Pyrimethamine	1 × 6	3	0		9
	5 × 6	3	0		11
Control	—	7	5	7-8	9-16

* Each chicken was inoculated with 1.2×10^3 sporozoites
† Day after inoculation
** Water suspension

*Table 6. Effect of QHS water suspension given ig on exoerythrocytic stage of P. cynomolgi**

Drug	Dosage MKD × days	Duration of administration	Parasitemia	Day of appearance
QHS	100 × 6	D_7-D_8	+	8
QHS	100 × 6		+	8
Primaquine†	3 × 6		+	22
CQ†	10 × 6		+	12
QHS	100 × 6	D_7-D_{13}	+	8
QHS	100 × 6		+	7
Primaquine†	3 × 6		—	
CQ†	10 × 6		+	8
Control	—		+	8

* Each monkey was inoculated with 8.7×10^6 sporozoites on D_0. Blood smears were made on D_7-D_{12}.
† Doses were calculated as base.

*Table 7. Effects of QHS (water suspension) and conventional antimalarials on the exoerythrocytic stage of P. yoelii**

Drug†	Dosage (mg/kg)	Therapeutic effect
Primaquine	30-60	Effective
Chloroguanide	5-10	Effective
Pyrimethamine	0.1-0.3	Effective
CQ	100	Non-effective
QHS	100	Non-effective

* Each mouse was inoculated with 2.5×10^6 sporozoites. Drug was given sc in single dose, 3-4 hours after inoculation. Blood smears were made on D_7-D_{14}.
† The drugs used were either dissolved or suspended in water according to their solubility.

Effect on Erythrocytic Stage of P. cynomolgi and P. knowlesi

Rhesus monkeys were inoculated intravenously with RBC infected with *P. cynomolgi*. When parasitemia reached a certain level after a latent period, the drugs were given intramuscularly once daily for 3 days; clearance and recrudescence times were determined by microscopic examination of blood smears at given intervals. Table 4 indicates that both QHS and artemether exhibited good antimalarial action against asexual forms of *P. cynomolgi*, artemether apparently exerting the better effect. In another experiment in which rhesus monkeys infected with *P. knowlesi* were given a daily dose of 6 mg/kg or more of sodium artesunate intravenously for 3 days, parasitemia disappeared within 16-20 hours after the first dose. No recrudescence was observed within 31 days.

Effect on Exoerythrocytic Stage of Plasmodia

1. Effect on the exoerythrocytic stage of *P. gallinaceum*.

Young leghorn chickens were inoculated with 1.2×10^3 sporozoites (isolated from *A. aegypti*) by intramuscular injection. Thirty minutes later, QHS (water suspension) was started intragastrically once a day for 6 days. Blood smears were made from D_2. Parasitemia, survival time and brain smears were examined. QHS 200 mg/kg given intragastrically once a day for 6 days showed no effect on the exoerythrocytic stage of *P. gallinaceum*. Chickens could not tolerate a daily dose of 400 mg/kg for 6 days (Table 5) and died within 7 days after start of medication.

2. Effect on the exoerythrocytic stage of *P. cynomolgi*.

Each monkey was inoculated with 8.7×10^6 sporozoites (isolated from *A. stephensi*) intravenously on D_0. QHS (water suspension) 100 mg/kg was given intragastrically from D_0 to D_5 or from D_5 to D_{10} qd for 6 days. Blood was smeared from D_7 on and parasitemia examined. Table 6 shows that parasitemia occurred on D_7 to D_8, indicating that the drug was not effective against the exoerythrocytic stage of *P. cynomolgi*.

3. Effect on the exoerythrocytic stage of *P. yoelii yoelii* (265 BY strain).

C_{57} black inbred mice (age 6-8 weeks) were inoculated intraperitoneally with 2.5×10^4 sporozoites (isolated from *A. stephensi*). 3-4 hours after inoculation, 100 mg/kg QHS (water suspension) was given by subcutaneous injection. Blood smears were made on D_7 and D_{14} to ascertain whether QHS could be used as causative prophylactic drug. Table 7 shows that QHS 100 mg/kg had no effect on the exoerythrocytic stage of *P. yoelii yoelii*.

4. Determination of effective concentrations of QHS, artemether and sodium artesunate on the erythrocytic stage of *P. falciparum* in cultures.

Conventional dose drug-sensitivity test as reported by Richards (1979) to determine the effective concentration of QHS against FCC2/HN strain revealed that QHS at concentration of $1 \times 10^{-7} - 10^{-6}$ M markedly inhibited the growth and multiplication of malaria parasites (Fig. 1). After cultivation in the medium containing QHS for 48 hours the parasitized RBC were transferred to normal medium without QHS, no increase in infection rate occurred. The in vitro minimum effective concentration was lower than that of CQ (1×10^{-6} M). QHS, artemether and sodium artesunate action on another strain of *P. falciparum* FCC1/HN was studied by microdrug-sensitivity test. 180 µl medium containing 2.5% (V/V) RBC (infection rate about 1%) and 20 µl of glucose saline containing drugs in different concentrations were added to wells of the plastic assay plate. CQ or blank was used as controls. After a culture period of 48 hours, smears were made from wells and examined under microscope. EC_{50} and EC_{90} of QHS, artemether and sodium artesunate calculated according to Finney's method were markedly lower than those of CQ, especially sodium artesunate (Table 8).

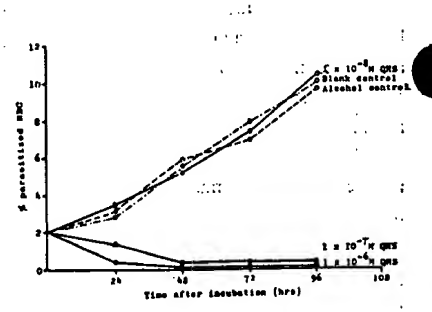

Fig.1 Effect of QHS on the growth of asexual form of *p. falciparum* in vitro (by Richards method)

Table 8. Effective concentration(ng/ml) of QHS and its derivatives acting on erythrocytic forms of FCCl/HN strain of P. falciparum in vitro

Drug	EC_{50}*	EC_{90}*	CQ equivalent	
			EC_{50}	EC_{90}
CQ	2.24	7.95	1	1
QHS	1.99	4.52	1.13	1.16
Artemether	2.19	4.12	1.02	1.96
Sodium artesunate	0.14	1.18	16.12	6.75

* EC_{50} or EC_{90} is the drug concentration which causes 50% or 90% reduction of the parasite density respectively.

PRELIMINARY STUDIES ON THE MODE OF ANTIMALARIAL ACTION OF QHS-TYPE COMPOUNDS

Morphological Studies

1. Effect of QHS on the ultrastructure of erythrocytic forms of *P. berghei* (in vivo).

QHS 100 mg/kg or 800 mg/kg, or CQ 40 mg/kg, was given. At intervals after medication blood samples were taken for examination under electronmicroscope. The asexual forms of the malaria parasites began to show pathological changes in 4-8 hours. In QHS groups, the swelling and spiral deformation of the membrane of food vacuoles, limiting membrane and the membrane of mitochondria appeared first, followed by swelling of the nuclear membrane and endoplasmic reticulum. In the CQ-treated group, autophagic vacuoles and clumping of malaria pigments were observed.

2. Effect of QHS on the ultrastructure of the erythrocytic form of *P. falciparum* (in vitro).

FCC2/HN CQ-sensitive strain of *P. falciparum* was cultured continuously by candle jar method. Drugs were added to the media and samples taken at definite intervals for electronmicroscopic examination. Under the action of QHS 1×10^{-7} M for 5×10^{-7} M for 2 hours or 1 hour respectively, morphological abnormalities could be observed in the asexual forms of *P. falciparum*. The main pathological changes were: 1) Injuries to the membrane structures of the parasite, such as swelling of the limiting membrane and the nuclear membrane. The intermembranous space widened, while the endoplasmic reticulum became swollen with a tendency to fuse together forming large autophagic vacuoles. Multilamellar bodies were seen in infected erythrocytes. No abnormal change was found in the membrane of mitochondria, a finding which differs from that obtained in *P. berghei* model. 2) Formation of autophagic vacuole. The autophagic vacuole contained elements of cytoplasm, but no malaria pigment was found. No correlation between the autophagic vacuole formation and membrane damage could be ascertained. Under the action of 1×10^{-6} M CQ the most prominent change was the formation of autophagic vacuole which contained not only cytoplasmic elements, but a large quantity of malaria pigments also. This is the basic difference between the autophagic vacuoles induced by CQ and QHS.

3. Effect of sodium artesunate on the ultrastructure of erythrocytic form of *P. knowlesi* (in vivo).

Two hours after sodium artesunate 10 mg/kg was injected intravenously into infected rhesus monkeys, the morphological changes of the membranes similar to those induced by QHS were observed in asexual forms of *P. knowlesi*.

The results mentioned above indicate that the morphological changes induced by QHS or sodium artesunate are quite different from those induced by CQ.

Biochemical and Pharmacological Studies

1. Effect of QHS on CQ-induced pigment clumping (CIPC).

Warhurst's method showed QHS as unable to induce pigment clumping, though the drug could somewhat inhibit CIPC. The maximal inhibition shown to be of non-competitive type was about 50%. The mode of action of QHS is therefore different from that of CQ.

2. The antagonism to the antimalarial action of QHS by PABA.

Experiments were performed in *P. berghei*-infected mice which were treated with QHS in 3-day regime. Results (Fig. 2) revealed that PABA did not suppress the antimalarial action of QHS but markedly antagonized the actions of SDM, TMP or pyrimethamine. The mode of action of QHS does not appear to be due to interference of folic acid metabolism in the parasites.

3. Effect of QHS on the incorporation of tritiated adenosine in erythrocytic forms of *P. berghei*.

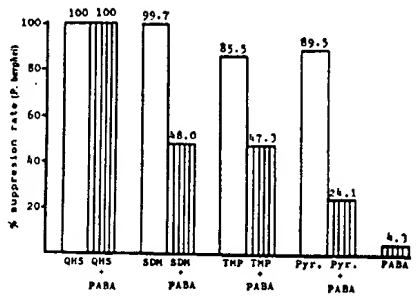

Fig. 2 Effect of PABA on the action of QHS and other antimalarial drugs

Table 9. Effect of QHS on the incorporation of tritiated adenosine by erythrocytic forms of P. berghei

Drug	Concentration (M)	Percentage of inhibition of incorporation	
		Parasitized RBC	Free parasite
Atabrin	1×10^{-4}	95±1.8	82
	1×10^{-5}	39±4.1	63
QHS	1×10^{-3}	15±12.7	7
	1×10^{-4}	9±4.4	0
	1×10^{-5}	1±1	

Effect of QHS on the incorporation of tritiated adenosine in parasitized RBC and free malaria parasites was studied by using Van Dyke's in vitro method. It was found that the incorporation of tritiated adenosine was markedly inhibited by 1×10^{-5} M atabrin, but almost not at all by QHS, even in a concentration of 1×10^{-3} M (Table 9).

4. Sodium artesunate had no effect on the synthesis of DNA in proliferating cells.

QHS-type compounds might not interfere with the nucleic acid metabolism of malaria parasites nor in such proliferating cells of mammals as spermatogonia in mice.

Studies are now under way on the effects of QHS and its derivatives on the respiration, carbohydrate metabolism and amino acid metabolism of malaria parasites.

SUMMARY

QHS, artemether and sodium artesunate showed good antimalarial action against the asexual forms of *P. berghei* and *P. cynomolgi*. The SD_{90} of the 3 drugs administered by intramuscular injection were 2.15, 0.53 and 1.77 MKD respectively. In comparison, the SD_{90} of CQ was 1.12 MKD. QHS was ineffective in treating the exoerythrocytic forms of *P. gallinaceum*, *P. cynomoigi* and *P. yoelii yoelii*.

su was effective in treating pernicious malaria but recrudescence rate was high, while intramuscular injection of the drug lowered the recrudescence rate. The first-pass effect of the drug is possibly another reason for the short duration of antimalarial action of orally administrated Qinghaosu. The rapid excretion of Qinghaosu and artemether plus the ineffectiveness of their metabolic products make the duration of action of these drugs short. Intramuscular injection is preferable in clinical practice. Since Qinghaosu may be detected in the brain and fetus, the embryonic and CNS toxicities of the drug may be understood.

SUMMARY

Following intravenous injection of Qinghaosu or artemether in rats and rabbits, the plasma drug concentration-time curves fitted to 2-compartment open models giving rise to short biologic half-lives ($t^1/_2\beta$ = 30.1 min for Qinghaosu, $t\frac{1}{2}\beta$ = 39.6 min for Artemether) and large apparent volumes of distribution (V_B = 4.1 L/kg for Qinghaosu, V_B = 3.0 L/kg for Artemether). These results indicate that Qinghaosu and Artemether were distributed widely in tissues and eliminated at a fairly rapid rate.

Absorption of Qinghaosu from the gastrointestinal tract in rats was found to be rapid and complete, but the plasma drug concentration was comparatively low and of short duration. In vitro experiments showed Qinghaosu to be readily metabolized by liver slices, indicating the presence of a first-pass effect. A large dose of the drug is required to obtain a blood level adequate for effective treatment of malaria when oral administration is the route selected.

A prolonged plasma level of Qinghaosu or artemether is obtained by intramuscular injection, which may be the preferred route to decrease the incidence of recrudescence in treating malaria. The bioavailability of intramuscularly injected Qinghaosu in aqueous suspension in rats was about 50% and that of oil suspension was much higher. Oil suspension of Qinghaosu is therefore preferred in the clinic. The bioavailability of intramuscularly injected artemether oil solution in rabbits was 37-50%.

Since sodium artesunate by intravenous injection was eliminated very rapidly from the body, intravenous drip may be preferable for maintaining adequate blood level of the drug in the treatment of cerebral malaria.

Appreciable levels of Qinghaosu, artemether and sodium artesunate by i.v. injection were found in the brain and fetus, indicating that these drugs can cross the blood-brain and blood-placenta barriers, a fact that may be relevant to the embryonic and CNS toxicities of the drugs.

Ethylacetate extraction yielded four kinds of crystals from the urine of Qinghaosu-treated patients. Three have been identified as deoxyartemisinine, dihydrodeoxyartemisinine and Crystal-7 all shown to be inactive against *P. berghei*. Other metabolites remain to be discovered.

REFERENCES

1. 中医研究院中药研究所药理研究室，北京师范大学生物系：^{3}H-青蒿素和^{3}H-还原青蒿素在小鼠在体内吸收、分布、排泄和代谢的初步研究。放射性同位素在基础医学中的应用，144～149页。《放射性同位素在基础医学中的应用》编辑组，原子能出版社，北京，1979。
2. Niu Xinyi, et al: The metabolic fate of Qinghaosu and TLC desitometric method for its determination in biological material. (To be published.)
3. Jiang JR, et al: Studies on metabolism of artemether. (To be published.)
4. Zeng YL, et al: Pharmacokinetics and bioavailability of artemether in rabbits. (To be published.)
5. 李锐等：青蒿素脂钠体内动力学研究。中草药(5)：20，1981。
6. 朱大元等：青蒿素生物代谢转化物的分离鉴定 I．人体代谢转化物的分离和鉴定。药学学报 15(8)，509，1980。

CORRECTION:

On page 84 of Vol. 1 No. 2, the picture in figure 2 should be in figure 3 while the one figure 3 should be in figure 2.

Journal of Traditional Chinese Medicine 2(1):31-38, 1982.

(2)

STUDIES ON THE TOXICITY OF QINGHAOSU AND ITS DERIVATIVES

*China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials**

This paper reports general and special toxicological experiments on Qinghaosu and its derivatives. Results point to the superiority of Qinghaosu and its derivatives over chloroquine in such respects as therapeutic index, margin of safety and side effects. It is pertinent therefore to recommend these compounds for clinical trial as antimalarials.

The main toxic effects of Qinghaosu were manifested on the hemopoietic system, especially the erythroid series, and the myocardium was somewhat involved. These toxic reactions were however reversible. The teratogenicity experiments in mice and rats showed both Qinghaosu and artemether to be embryo-toxic, and clinical trials should take into account these toxic effects. Further studies with various other animal species should be aimed at discovering whether species differences exist.

Studies on the antimalarial effect of Qinghaosu and its derivatives artemether and sodium artesunate demonstrated definite therapeutic effect on the erythrocytic stage of Plasmodia. In order to provide reference data for the safe clinical use of these drugs, a series of toxicological studies on the drugs was carried out.

TOXICOLOGICAL STUDIES ON QINGHAOSU

General Toxicity

1. Acute toxicity

a) Toxicity of single dose to mice, rats and dogs:

Hybrid Kunming strain mice, 20 ± 2 grams, of both sexes, and Wistar rats around 200 grams were used. Qinghaosu, particle-size less than 15μ, was administered either per os (water suspension), or intramuscularly (oil suspension).

The LD_{50} and the chemotherapeutic index (LD_{50}/SD_{50}) of Qinghaosu were determined by the Miller and Tainter Method. The results (Table 1) showed both to be greater than those of chloroquine in mice whether by oral or intramuscular administration.

Water suspension of Qinghaosu, 800 and 400 mg/kg, was given in single doses to 2

Table 1. Acute toxicity of Qinghaosu in single dose to mice and rats

Animal species	Drugs	Route of administration	LD_{50} (mg/kg)	Chemotherapeutic index
Mouse	Qinghaosu	per os	4228	384
	Chloroquine	per os	400	216
	Qinghaosu*	i.m.	3840	4987
	Chloroquine	i.m.	63	95
Rat	Qinghaosu	per os	5576	...
	Qinghaosu*	i.m.	2571	...

Note: * oil suspension of Qinghaosu.

* Main research units:

1. Institute of Chinese Materia Medica, Academy of Traditional Chinese Medicine
2. Academy of Military Medical Sciences
3. Institute of Traditional Chinese Medicine and Materia Medica, Shandong Province
4. Institute of Materia Medica, Yunnan Province
5. Institute of Materia Medica, Sichuan Province
6. Shanghai Institute of Materia Medica, Chinese Academy of Sciences
7. Guangzhou Traditional Chinese Medical College
8. Guangxi Medical College
9. Guangxi Traditional Chinese Medical College

Exhibit 2

dogs respectively by intramuscular injection. No death occurred within the 10-day period of observation. About 15 minutes after injection the dog receiving the large dose developed tonic and clonic convulsion and even episthotonos, while the dog receiving the small dose became excited and howled. These symptoms subsided spontaneously after about 30 minutes. 48 hours after injection a significant decrease in reticulocyte count was found, plus a slight increase in SGPT and SAP activity, the changes being more pronounced in the large-dose dog. Most of the changes returned to normal on the 10th day after injection. Histopathological examination of viscera and bone marrow revealed no significant difference between the experimental animals and the vehicle controls.

b) Symptoms and signs of acute intoxication:

For systematic observation of acute intoxication manifestations of Qinghaosu, large single doses of the drug were given intramuscularly to several species of animals, followed by doubled doses after a certain period. The animals generally developed the following symptoms and signs: some restlessness, tremor and incoordination followed by inhibited activity, slow respiration, delayed sensation and disappearance of righting reflex. The smaller animals showed no apparent nervous system symptoms, but such larger animals as pigeon, guinea pig, rabbit, cat and dog demonstrated clonic and tonic convulsions, and even episthotonos. Respiration always ceased before cardiac arrest, and autopsy revealed that cardiac contraction had stopped at systolic phase. Species difference exists in the susceptibility of animals to the toxicity of Qinghaosu, pigeons being the most sensitive and rats the most tolerant to the drug. Surviving animals returned gradually to normal within about 10-24 hours.

Further investigation is necessary to explain cause of death of animals during acute intoxication.

c) Acute toxicity of repeated medication within a short period:

40 rats weighing 200 to 220 grams each were divided into four groups of 10. Three groups had respectively 600, 400, and 200mg/kg of Qinghaosu in oil suspension injected intramuscularly once daily for 7 consecutive days. The fourth group was injected with the same volume of oil and served as controls. Body weight, food consumption and serum transaminase activity were observed before and after injection. Pathological examinations were also made. No remarkable changes were found except for some engorgement and slight degeneration of heart, liver, spleen, lung and kidney in the large- and medium-dose groups. No death due to acute intoxication occurred.

Four dogs weighing 6.7 to 10.0 kg were given 100 mg/kg of Qinghaosu per os daily for five successive days. No apparent intoxication reaction or appreciable change in respiration, cardiac rate or cardiac rhythm was observed.

2. Subacute toxicity

Test for subacute toxicity of Qinghoasu was carried out according to the WHO recommendation.[1]

a) Subacute toxicity of Qinghaosu given per os to rats:

32 rats weighing 80-100 grams were divided into four groups of 8. Three groups were given respectively 250, 500 and 1000 mg/kg of Qinghaosu intragastrically once daily for 14 successive days, the fourth group serving as control. Body weight, ECG and routine blood tests were recorded before administration of the drug, and ECG was taken 1 to 2 hours after the last dose. Blood and urine samples were obtained for routine analysis 24 hours after completion of medication, when half of the animals in each group were sacrificed; heart, liver, spleen, lung, kidney, brain and stomach were examined for pathological changes. The other half of the animals were sacrificed one week later and the same examinations made. No significant difference was found between the treated and control groups.

b) Subacute toxicity of Qinghaosu injected intramuscularly to monkeys:

An equal number of male and female rhesus monkeys from Yunnan Province weighing 3 to 6 kg and of 4 to 7 years of age were used after group feeding for one year. The animals were caged separately during experimentation. Details of grouping and treatment are listed in Table 2. Observations and determinations were as follows:

Table 2. Grouping and management of monkeys for subacute toxicity studies

Group No.	Dose (MKD)*	No. of animals		No. of animals sacrificed 3 days after last dose	No. of animals evaluated for reversibility
		female	male		
I	192	2	2	4	...
II	96	3	3	4	2
III	48	3	3	4	2
IV	24	2	2	4	...
V	Vehicle control**	3	3	4	2

Notes: * mg/kg/day, given im for 14 consecutive days.
** The vehicle consisted of 0.4% phenol (V/V) and 2% benzyl alcohol (V/V) in peanut oil. The volume of the vehicle injected was the same as for group I.

Clinical observation included behavior, appetite, body temperature, body weight, nausea and vomiting, stools, and local reaction at injection sites.

Urinalysis and microscopic examination of sediment: pH, color, sugar, protein, ketone bodies, bilirubin, urobilinogen, nitrous compounds, WBC and RBC, blood, epithelial cells, salts.

Hematology: platelets count, thromboelastogram, reticulocyte count, RBC, Hb, hematocrit, ESR, total and differential WBC counts.

Bone marrow smear examination: differential count, the ratio of myeloid to erythroid series, the ratio of immature to mature cells and mitotic index.

Blood biochemical analysis: protein electrophoresis, BUN, CRTN, CPK, LDH, GOT, GPT, SAP, TBil, TP, Glu, TriG, Chol, Ca, P, K, Na, corticosterol and corticosterone.

Pathological examination: 52 samples taken from various organs, tissues and glands were examined. Besides the routine hematoxylineosine stain, fat stain was used on sections of heart, liver, kidney and adrenal gland. Electronmicroscopy was made on heart, liver, kidney and bone marrow. Special stains were used for hemosiderin, collagen and bilirubin.

Details of these results are described elsewhere in reports and only a brief account follows.

Qinghaosu (oil suspension) 192 MKD injected intramuscularly for 14 successive days was highly toxic to monkeys, causing 3 deaths among 4 animals within 3 days after the last dose. The main manifestations of toxic effects were reduced appetite, apathy, decreased activity and slowing of cardiac rate. Reticulocytes in peripheral blood disappeared, RBC, hematocrit and Hb were all decreased, while ESR increased. The total number of WBC and percentage of neutrophils decreased, while the number of platelets increased slightly. Bone marrow smears showed profound inhibition in hemopoietic function, especially the erythroid series. The ratio of myeloid to erythroid series increased, while that of immature to mature erythroid cells decreased. Biochemical analysis of blood revealed remarkable decrease of cortisol content. A tendency of increase of CPK and GOT activities and of BUN, triglyceride, cholesterol, inorganic phosphate and K^+ content as well as a tendency for blood sugar and Na^+ to decrease were also observed. Histopathological examination by light and electron-microscopes of various organs of animals that died naturally or were sacrificed showed definite cytoplasmic coagulation and mitochondrial swelling in cardiac muscles, slight cloudy swelling of epithelial cells of renal tubules, and slight accumulation of glycogen and vacuolar degeneration in liver parenchymal cells. The findings in bone marrow sections were the same as in smears.

Qinghaosu 96 MKD injected intramuscularly for 14 successive days also had severe toxic effects similar to those described above.

One of six injected animals died 2 days after the last dose.

No animal given Qinghaosu 48 MKD showed apparent abnormalities, though laboratory examination revealed disappearance of reticulocytes, decrease in RBC and Hb content, slight decrease of packed cell volume and some increase of ESR. No obvious changes were found in smears and histological sections of bone marrow. Neither clinical chemistry nor histopathological examination revealed any significant change.

Decrease of reticulocytes was the only change found in the group given 24 MKD.

All positive findings in two monkeys in each of the 48- and 96-MKD groups returned to normal within 22 days after withdrawal of the drug.

The above results indicate that Qinghaosu (oil suspension) at doses below 24 MKD injected for 14 successive days were safe for monkeys; doses above 48 MKD were harmful, and doses larger than 96 MKD were lethal.

Apparently the toxic effect was mainly manifested on the hemopoietic cells of bone marrow, especially the erythroid series. The myocardium was found to be somewhat involved also. These toxic reactions were reversible however.

Further studies elucidating the cause of death after intoxication are needed.

Special Toxicity

1. Musculo-irritant Test

The method for testing musculo-irritant effects of drugs recommended by the American Association of Pharmaceutical Industry[2] was adopted. The animals used were Japanese long-ear albino rabbits. The injection volume of oil or water suspension was 1 ml containing 50 mg of Qinghaosu. The injection sites were examined both macroscopically and microscopically. Results were expressed in standard scores. Serum CPK was determined by simplified Okinaka's method before and after injection. The results gave scores in pathological examination of both the Qinghaosu-injected and the control group all below 6, while the SCPK activities after medication were all below 2000 iu/L, indicating no noticeable local injury caused by these two preparations.

2. Mutagenicity Studies of Qinghaosu

The test for micronucleus of polychromatic erythrocytes of mammalian bone marrow and Ames method were adopted for detecting the mutagenicity of Qinghaosu.

a) Murine bone marrow polychromatic erythrocyte micronucleus test:

Following Schmid's method[3], hybrid Kunming strain mice were used and 1/80, 1/40, 1/20, 1/10 or 1/5 of the LD_{50} (4228 mg/kg) of Qinghaosu was given to five groups of animals. Results showed no effect on the frequency of micronucleus of mouse bone marrow polychromatic erythrocytes by Qinghaosu, probably indicating that the drug did not mutagenically affect the mammalian in vivo system.

b) Ames test

Following Ames Salmonella/mammalian microsome enzyme test method[4] and referring to Takin Yahagi's improved vibrating incubation procedure[5], the mutagenic effect of Qinghaosu was determined quantitatively. Qinghaosu was dissolved in DMSO to the concentrations of 300, 30, 3, 0.3 and 0.03 μg/0.1 ml. Negative results indicated that Qinghaosu is not mutagenic.

c) Teratogenicity studies on Qinghaosu

Referring to Wilson's method, Qinghaosu was given orally to pregnant Wistar rats at doses of 1/400, 1/200 or 1/25 of LD_{50} in order to observe its effect at different dosages on fetal rats at different periods of gestation.

Most of the fetal rats survived the 1/25 LD_{50} dose of Qinghaosu when given during the first 6 days of gestation, all the living fetus developing normally and without deformity, indicating that the drug had little effect on the development of fetal rats early in gestation. When Qinghaosu was given in mid and late gestation (7th to 12th day and 13th to 19th day), no fetus survived, indicating high toxicity to the fetal rats at these periods of gestation.

When 1/200 or 1/25 of LD_{50} of Qinghaosu was given to pregnant rats on the 6-15th day of gestation, 100% of the fetuses were absorbed, while at the lower dose of 1/400 of the LD_{50} about half were absorbed. When the period of organogenesis was further divided into early (6th to 8th day), middle (9th to 11th day) and late (12th to 14th day) stages, Qinghaosu at a dose of 1/25 of LD_{50} given in the early stage caused the deformity of umbilical hernia in 6.1% of the rat fetuses, while the rest were normal. Qinghaosu given in the middle and late stages of organogenesis resulted in absorption of all the fetuses.

Experimental results in mice were similar, showing evident toxicity of Qinghaosu to both the mouse and rat fetus, especially during the mid and late periods of gestation. This demands attention. Further studies are needed to determine whether Qinghaosu exhibits similar teratogenic effects on animals other than rodents.

TOXICITY OF ARTEMETHER

Acute Toxicity

1. Mouse:

Mice were given a single dose of artemether and observed for 7 days. The LD_{50} of artemether calculated according to Finney's method was 263 mg/kg for intramuscular injection. The therapeutic index was 447.

All animals showed the following symptoms and signs: mental sluggishness, quiet lying posture, refusal to take food, hair standing up. Heart rate slowed before the animals succumbed.

2. Dog:

A bitch weighing 13 kg was given a 130 mg/kg dose of artemether intragastrically, followed by a second dose two days later. No toxic effect such as vomiting was observed.

3. Monkey:

A single dose of artemether 141 mg/kg was given intramuscularly to a male monkey weighing 7 kg and was well tolerated.

4. Rabbit:

Single dose of artemether 160 mg/kg was injected intramuscularly to several rabbits weighing 2.6 ± 0.96 kg. All the animals tolerated the drug well.

Subacute Toxicity

1. Rat:

Total doses of 40 to 360 mg/kg of artemether were injected intramuscularly to rats within a 9 to 14-day period. Loss of body weight in the higher dose group occurred. Histopathological examinations showed the

only change of toxicological importance to be slight fatty degeneration in liver cells.

2. Dogs:

A first course of artemether was given to one group of dogs intramuscularly once daily for 3 days, a second course being given after a 7-day interval. The same regimen of artemether was applied to another group of dogs for 3 months. Total doses given these two groups were 24 and 972 mg/kg respectively. The animals were examined during medication, the items consisting of general observations, blood and urine routine tests, biochemical analysis of blood, ECG and histopathological examinations on heart, liver, spleen, lung, kidney, brain, adrenal gland, hypophysis, gonads and other organs. Results were all negative except for loss of body weight and slight fatty degeneration of liver cells in the higher dose group.

3. Monkey:

Total doses of 97 and 292 mg/kg of artemether were injected intramuscularly in the course of 1 to 3 months, with no abnormal changes found.

Local Irritation

No musculo-irritant effect was observed when artemether was injected intramuscularly to mice, rats, rabbits, dogs and monkeys. Examination of histological section showed normal muscle fibers. No obvious increase of SCPK was found after injection of the drug.

Teratogenicity Experiments

Artemether is highly toxic to mouse and rat embryos. Intramuscular injection of 10.72 mg/kg (1/25 of LD_{50}) of artemether in the course of 10 days to female mice between the 6th and 15th days of gestation (period of organogenesis) caused absorption of 30.1% of the fetus. Some survived, however, and developed well without deformity. When a dose of 21.44 mg/kg was given at different periods after fertilization, maximum effect was observed between the 9th and 11th day of gestation. Artemether at doses larger than 21.44 mg/kg killed all fetuses and 100% fetal absorption resulted. Its effect on rats was the same.

TOXICITY OF SODIUM ARTESUNATE

Acute Toxicity

1. Mouse:

The LD_{50} of sodium artesunate was 520 mg/kg for intravenous, and 475 mg/kg for intramuscular injection. The therapeutic index (iv) was 1733 for sensitive strain and 1040 for chloroquine-resistant strain of *P. berghei*.

2. Dog:

Sodium artesunate was injected intravenously to three dogs with increasing doses, i.e. 37.5 mg/kg on the first day, 75 mg/kg on the second, and 100 mg/kg on the third. All dogs ingested less food after injection of the second dose and vomited one to five times during one hour, beginning about ten minutes after the third injection. The dogs lay down, refused to eat and looked exhausted, these latter symptoms persisting for 2 days before full recovery.

3. Effect on heart rate, cardiomuscular tone and coronary blood flow of the guinea pig heart in vitro:

The cardiomuscular tone was inhibited when the sodium artesunate concentration reached 1×10^{-3}. Cardiac rate and coronary blood flow were not affected.

4. Cardiovascular effects of rapid intravenous injection on anesthetized rabbit:

Blood pressure and ECG (second lead) were recorded for three rabbits anesthetized with intravenous urethan 1 g/kg. Sodium artesunate dissolved in normal saline at a dose of 100 mg/kg was injected rapidly into the marginal vein of the ear once every 2 minutes. Each injection was completed within 3 to 5 seconds. The accumulated dose was recorded when significant changes in blood pressure, ECG or death of each animal occurred. Results showed that slowing of cardiac rate, prolongation of P-P interval and decrease of blood pressure began when the accumulated dose reached 300 mg/kg. Marked delay of sinus rhythm, reduction of heart rate to one half and decrease of blood pressure to 40 mmHg occurred as the accumulated dose reached 600 mg/kg. Flattened, biphasic even inverted T wave appeared at 800 mg/kg, and cardiac arrest and death occurred when the accumulated dose reached 1200 mg/kg. In contrast, when CQ was given to the animals the blood pressure dropped to 40 mmHg when the accumulated dose reached 11.2 mg/kg and cardiac arrest occurred at 22.8 mg/kg. The cardiovascular toxic effect of sodium artesunate was 53.6 times lower than that of chloroquine with the dosage required to decrease the blood pressure to 40 mmHg used as criterion.

Subacute Toxicity

1. Dog:

Six dogs were evenly divided into 3 groups. Two groups were injected intravenously with 10 and 40 mg/kg of sodium artesunate once daily for 14 days. The third group served as control with the same volume of normal saline injected.

Body weight, appetite, stool, urine, behavior, blood routine test, SGPT, NPN were examined before and after injection of the drug with no abnormal changes found. Autopsy was performed the day after the last injection. Small petechiae or congestion of liver was observed in one dog of each group, but there were no other positive findings.

2. Monkey:

Six monkeys were divided into three groups of 2. Two groups received 10 and 32 mg/kg of sodium artesunate by intravenous injection once a day for 14 days. The third group were injected with the same volume of normal saline as control.

Autopsy was performed the day after the last injection and no abnormal changes were found, using the same observation items as for dogs.

Local Irritation Test

No irritation caused by the drug was found on gross and microscopic examinations of muscles and blood vessels at the site of injection, iv or im, in rabbits, dogs and monkeys.

SUMMARY

This paper presents the results of our recent toxicological studies on Qinghaosu and its derivatives artemether and sodium artesunate.

The toxicity of Qinghaosu, artemether and sodium artesunate was far less than that of chloroquine (CQ), while the therapeutic index of the three drugs studied was

Recent progress of malaria research: chemotherapy

Mechanism of action of antimalarial drugs
Folate pathway antagonists
— Sulfonamides and sulfones
— Dihydrofolate reductase inhibitors
Chloroquine and related blood schizontocides
Antibiotics
Qinghaosu, its derivatives and other plant-derived products
Naphthoquinones
Tissue schizontocides

Mechanisms of drug resistance
Resistance to dihydrofolate reductase inhibitors
Resistance to sulfonamides and sulfones
Resistance to 4-aminoquinolines
Resistance to quinine and mefloquine
Resistance to Qinghaosu and its derivatives
Resistance to antibiotics
Resistance to primaquine

Pharmacokinetics
4-aminoquinolines
Primaquine
Quinine
Proguanil
Sulfadoxine and pyrimethamine
Mefloquine

Repository drugs
Inherently long-acting formulations
Chemical modification of drugs to extend duration of action
Delayed degradation and excretion of antimalarial drugs
Sustained release formulations
Screens for repository formulations

Targeting of drugs

Chemotherapeutic approaches based on parasite biochemistry
Biochemical targets
— Energy metabolism
— Protein synthesis
— Nucleic acid synthesis
— Folate metabolism
— Lipid biosynthesis
Microtubules
Parasite invasion of red cells
Oxidant killing of malaria parasites

Exploitation of potential biochemical targets for drug action

New candidate antimalarials
Candidate antimalarials in an advanced state
— 9-phenanthrenemethanols
— Sesquiterpene lactones
— Pyronaridine
— Enpiroline
Candidate antimalarials in an advanced preclinical state
— 4-aminoquinolines and Mannich bases
— 8-aminoquinolines
— 4-quinolinemethanols
— Quinolones
— Naphthoquinones
— Quinazolines
— Dihydrotriazines
Other compounds of interest

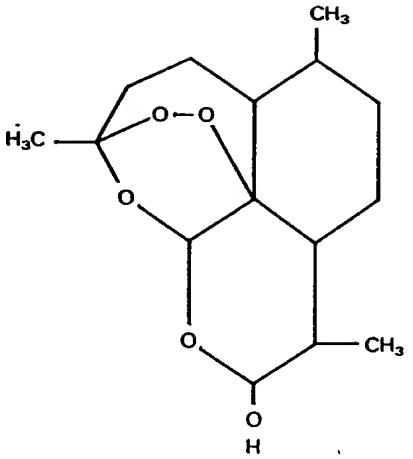

Fig. 51.6 Dihydroqinghaosu

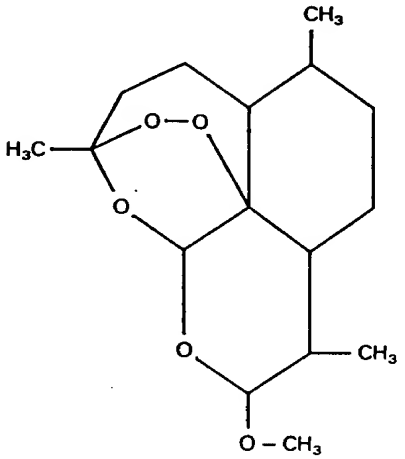

Fig. 51.7 Artemether

soluble in water, but rather unstable in aqueous solution. Sodium artesunate is highly hygroscopic, posing major problems in the formulation of the compound. The free acid (artesunic acid or Qinghaosu succinic acid) is not hygroscopic, and is as effective as the sodium salt.

It is to be expected that Qinghaosu or its derivatives may become drugs for the treatment of malaria, especially of forms requiring rapid medication such as hyperacute or complicated falciparum malaria. However, the final selection of a candidate analogue will probably be made on the basis of the structure/activity relationships

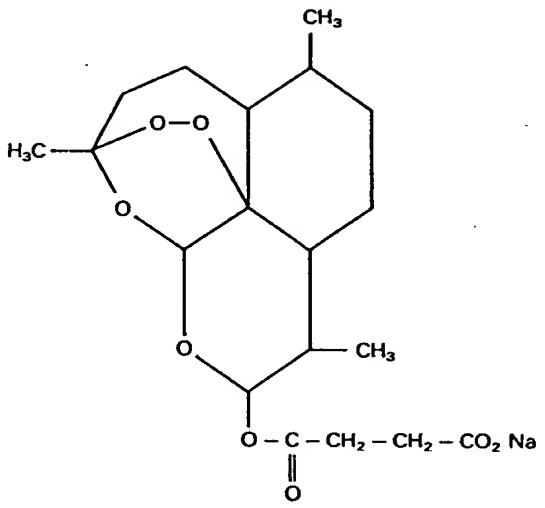

Fig. 51.8 Na-artesunate

already elucidated by Wu & Ji (1982), and of the economics of the synthesis of derivatives using Qinghaosu isolated from the plant. The selection will also be influenced by the envisaged use of the drug. A compound lending itself to the formulation for i.m. injection in relatively small volumes is apt to receive preference, as it would facilitate emergency treatment of malaria at a relatively low level of the primary health care system.

Animal and in vitro studies

The SD_{50} and SD_{90} (SD = suppressive dose) of Qinghaosu, artemether and sodium artesunate were determined by comparison with chloroquine in hybrid Shanghai mice intraperitoneally inoculated with 5×10^6 *P. berghei*-infected erythrocytes (CCRG 1982c), using a chloroquine-sensitive isolate. The drugs were given on days 1, 2 and 3, in one daily dose. The blood was examined on day 4. The results shown in Table 51.3 indicate that artemether in oil solution had the highest dose efficacy, and the least proportional difference between SD_{50} and SD_{90}. The marked difference between the activity of the water suspension and the oil suspension of Qinghaosu, both given by the i.m. route, is apparently due to its solubility and absorption characteristics. The difference

Table 51.3 Antimalarial activity of Qinghaosu and its derivatives in mice infected with chloroquine-sensitive *Plasmodium berghei* (based on data from CCRG 1982c)

	SD_{50} mg/kg	SD_{90} mg/kg	Ratio of SD_{90}:SD_{50}
Qinghaosu, water suspension, i.g.	10.80	28.30	2.62
Qinghaosu, water suspension, i.m.	4.90	8.01	1.63
Qinghaosu, oily suspension, i.m.	0.77	2.15	2.79
Artemether, oily solution, i.m.	0.37	0.53	1.43
Na-artesunate, water solution, i.m.	0.54	1.77	3.28
Na-artesunate, water solution, i.v.	0.94	3.10	3.29
Chloroquine, water solution, i.g.	1.85	2.60	1.41
Chloroquine, water solution, i.m.	0.60	1.12	1.87
Chloroquine, water solution, i.v.	0.67	1.25	1.87

of activity of the water suspension given by the intragastric or the intramuscular route may indicate poor gastrointestinal absorption or degradation before absorption, but it is more likely to be due to a first pass effect. Using the same *P. berghei* isolate and treating the mice once a day for three days as soon as parasitaemia had reached 5 ± 2%, Qinghaosu, artemether, sodium artesunate and chloroquine were given in order to assess equi-effective doses and the speed with which parasitaemia was reduced. Qinghaosu and its derivatives all cleared parasitaemia faster than chloroquine, with sodium artesunate exhibiting the fastest effect but the highest incidence of recrudescences (CCRG 1982c).

Qinghaosu and artemether proved to be highly effective also in mice infected with a chloroquine-resistant isolate of *P. berghei*, but there was a difference in the dose response between the chloroquine-sensitive and the chloroquine-resistant isolate. The resistance index, i.e. the ratio between ED_{50} or SD_{50} levels of resistant and sensitive isolates was 3.6 for Qinghaosu (water suspension, intragastric), 1.7 for artemether (oily solution, i.m.) and sodium artesunate (water solution, i.v.) against an index of 52 for chloroquine.

Qinghaosu has also been tested independently by the Walter Reed Army Institute of Research in a rodent model and against *P. falciparum* in vitro. In the *P. berghei* schizontocidal test it was inactive after oral as well as subcutaneous administration of up to 80 mg/kg, the highest tested dose. However, it was effective subcutaneously in suppressive testing against both the chloroquine-sensitive and -resistant lines (SD_{90} 22 mg/kg per day against the sensitive strain and 31 mg/kg per day against the resistant strain). In vitro against *P. falciparum*, it was effective against both the Camp and the Vietnam Smith strains with an EC_{50} of 0.42 and 0.23 ng/ml respectively (Klayman et al 1984a). Thus, no cross-resistance with chloroquine was observed. These results agree with those reported by Chinese scientists and indicate that the drug is poorly active orally.

Macaca mulatta, intravenously infected with blood stages of *P. cynomolgi*, were given Qinghaosu and artemether at various dose levels for three days after parasitaemia had reached full patency. Qinghaosu, administered i.m. as an oily suspension, produced cure at 20 mg/kg body weight once daily for three days; all animals treated with 10 mg/kg or less showed recrudescences. With artemether, administered i.m. as an oily solution, the dose of 8 mg/kg daily for three days proved to be curative; 4 mg/kg was not always curative and recrudescences occurred in all animals having received a lesser dose. Sodium artesunate (water solution i.v.) acted very quickly and was radically curative in *P. knowlesi*-infected *Macaca mulatta* when given at a daily dose of 6 mg/kg or more for three days (CCRG 1982c).

Qinghaosu had no effect against the exoerythrocytic stages in sporozoite-infected chickens (*P. gallinaceum*), mice (*P. yoelii yoelii*) and rhesus monkeys (*P. cynomolgi*).

Qinghaosu proved to be parasitocidal at concentrations $\geq 10^{-7}$ mol/l when tested in vitro according to the technique of Richards & Maples (1979), using the FCC1 and FCC2 isolates of *P. falciparum* from Hainan (CCRG 1982c). In

MARTINDALE

The Extra Pharmacopoeia

Thirty-first Edition

Edited by James E F Reynolds

Deputy Editor
Kathleen Parfitt

Assistant Editors
Anne V Parsons
Sean C Sweetman

Published by direction of the Council of the Royal Pharmaceutical Society of Great Britain and prepared in the Society's Publications Department

London
ROYAL PHARMACEUTICAL SOCIETY
1996

J thickening agent and, as silica gel, as a des-

dal silicon dioxide is used as a suspending
nd thickener, as a stabiliser in emulsions, and
nticaking agent and desiccant.

n dioxide (Silicea) is used in homoeopathic
ne.

ged inhalation of some forms of silica dust
associated with the development of fibrosis
(silicosis) or with cancer. However, these
of silica used as pharmaceutical excipients do
pear to be associated with silicosis.

arations

preparations are listed below; details are given in Part 3.

etary Preparations

Celloid S 79; *Ger.:* Aktiv-Puder; Entero-Teknosal; Skle-
n.: Dissolvurol.

ngredient preparations. *Aust.:* Kephalodoron; *Aus-*
-Disc; Duo Celloid SCF; Duo Celloid SPS; Duo Celloid
nd Nail Formula†; *Belg.:* Trisibam†; *Canad.:* Topol
Fluoride; Topol with Fluoride; *Fr.:* Gastralugel; Gastro-
Gel ...se; Topaal; Urémiase; *Ger.:* Adsorgan†; Aplo-
...salbe†; CO- Granulat; Decoderm Basiscreme;
rment-N†; Equisil; Gastrovison†; Presselin Olin 1†; Ro-
Siozwot; Tectivit†; Vobaderm Basiscreme†; *Ital.:* Bel-
trovirson; Lacalut; *Mon.:* Dissolvurol†; *Spain:* Sales Gras;
Vioplastine poudre†; Balsafissan; Cicafissan; Fissan; Glo-
K: Bidor; WCS Dusting Powder.

ium Starch Glycollate (5460-y)

Carboxymethyl Starch; Sodium Starch Glycolate;
odium Glycollate.

9063-38-1.

opoeias. In *Chin., Fr.,* and *It.* Also in *USNF.*
Eur. include Sodium Starch Glycollate (Type A) and So-
arch Glycollate (Type B).
ndards of *Ph. Eur.* apply to those countries that are par-
e Convention on the Elaboration of a European Phar-
eia, see p.xiii.

ium salt of a carboxymethyl ether of starch.

Starch Glycollate (Type A) (BP 1993) and Sodium
lycolate (USNF 18) contain 2.8 to 4.2% of sodium.
Starch Glycollate (Type B) (BP 1993) contains 2.0 to
odium.

odourless, white or almost white, very hygroscopic,
ing powder. Practically insoluble in methylene chlo-
ms a translucent suspension in water. A 2% disper-
cold water settles, on standing, to give a highly
lav... Store in airtight containers. Protect from light,
...ns in temperature and humidity which may
aking.

n starch glycollate is used as a disintegrating
n tablet manufacture.

Tragacanth (5463-c)

E413; Goma Alcatira; Gomme Adragante; Gum Dragon; Gum Tragacanth; Trag.; Tragacantha; Tragacanto; Tragant.

CAS — 9000-65-1.

Pharmacopoeias. In *Aust., Belg., Br., Cz., Eur., Fr., Ger., It., Jpn, Neth., Port.,* and *Swiss.* Also in *USNF.*
The standards of *Ph. Eur.* apply to those countries that are parties to the Convention on the Elaboration of a European Pharmacopoeia, see p.xiii.

The dried gummy exudation flowing naturally or obtained by incision from the trunk and branches of *Astragalus gummifer* and some other species of *Astragalus* (Leguminosae) from western Asia.

It occurs as thin, flattened, more or less curved, ribbon-like, white or pale yellow, translucent, horny odourless strips.

The powder forms a mucilaginous gel with about ten times its weight of water.

Store in airtight containers. Protect from light.

Powdered Tragacanth, which is specified in the BP and USNF, is a white, almost white, or yellowish white powder.

Adverse Effects

Hypersensitivity reactions, sometimes severe, have occurred rarely after the ingestion of products containing tragacanth. Contact dermatitis has been reported following the external use of tragacanth.

Uses

Tragacanth forms viscous solutions or gels with water, depending on the concentration. In dispensing aqueous preparations of tragacanth, the powdered tragacanth is first dispersed in a wetting agent, such as alcohol, to prevent agglomeration on the addition of water.

Tragacanth is used as a suspending agent and as an emulsifying agent. It is also used for these purposes in the food industry.

An acceptable daily intake for tragacanth as a food additive was not specified as the total daily intake arising from its use at the levels necessary to achieve the desired effect, and from its acceptable background in food, was not considered to represent a hazard to health.[1]

1. FAO/WHO. Evaluation of certain food additives and contaminants: twenty-ninth report of the joint FAO/WHO expert committee on food additives. *WHO Tech Rep Ser 733* 1986.

The FDA has advised that preparations containing compounds such as tragacanth that may be taken by mouth in bulk laxatives or weight-control preparations should be taken with a full glass of water or, if the patient has difficulty in swallowing, they should be avoided. Such compounds swell into masses that may obstruct the oesophagus if not taken with sufficient water.

Preparations

Names of preparations are listed below; details are given in Part 3.

Official Preparations

BPC 1973: Tragacanth Mucilage.

Proprietary Preparations

Multi-ingredient preparations. *Ital.:* Normacol†.

Xanthan Gum (5465-a)

Corn Sugar Gum; E415; Polysaccharide B 1459; Xantham Gum.

CAS — 11138-66-2.

Pharmacopoeias. In *Fr.* Also in *USNF.*

A gum produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris* and purified. It is the sodium, potassium, or calcium salt of a high molecular weight polysaccharide containing D-glucose, D-mannose, and D-glucuronic acid. It also contains not less than 1.5% of pyruvic acid.

A cream-coloured powder. **Soluble** in hot and cold water. A solution in water is neutral to litmus.

Uses

Xanthan gum is used as a stabiliser, thickener, and emulsifier. It is also used similarly in the food industry.

An estimated acceptable daily intake of xanthan gum is up to 10 mg per kg body-weight.[1]

1. FAO/WHO. Evaluation of certain food additives and contaminants: twenty-ninth report of the joint FAO/WHO expert committee on food additives. *WHO Tech Rep Ser 733* 1986.

The FDA has advised that preparations containing compounds such as xanthan gum that may be taken by mouth in bulk laxatives or weight-control preparations should be taken with a full glass of water or, if the patient has difficulty in swallowing, they should be avoided. Such compounds swell into masses that may obstruct the oesophagus if not taken with sufficient water.

Suspensions of crushed tablets or insoluble powders made with xanthan gum were reported to be preferable to those made with tragacanth.[1]

The stability was generally good and only a small number of drugs had been found to be incompatible (amitriptyline, tamoxifen, and verapamil).[1] For extemporaneous dispensing, a 1% solution of xanthan gum with hydroxybenzoate, prepared in advance, was diluted to 0.5% with water when preparing the suspension.

Xanthan gum was found to be a suitable suspending vehicle for delivering antispasmodics topically along the length of the oesophagus in patients with oesophageal spasm.[2] Coagulation of the gum had been observed when it was used for suspensions of certain film-coated tablets.

1. Anonymous. "Extremely useful" new suspending agent. *Pharm J* 1986; 237: 665.
2. Evans BK, Fenton-May V, Keltrol. *Pharm J* 1986; 237: 736–7.

Preparations

Names of preparations are listed below; details are given in Part 3.

Proprietary Preparations

Multi-ingredient preparations. *UK:* Magnatol.